ISOLATION OF BETA 1, 3 GLUCAN FROM *SACCHAROMYCES CEREVISAE* : AN EXPERIMENTAL STUDY ON ITS ADJUVANTICITY

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(Hemant Kumar)

ABSTRACT

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The present study was aimed to isolate β-1,3 Glucan, a inner cell wall component from compressed bakers yeast, Saccharomyces cerevisae. These studies were also carried out to evaluate the toxicity in an experimental animal and to further assess the adjuvanticity of β-1,3 Glucan, which is commonly employed as an immunomodulator in vaccination studies against a large number of parasitic infections. In this study β-1,3 Glucan was isolated as per modified method of Di Luzio et al (1979) and Hassid et al (1941). The various procedures employed for glucan extraction enabled us to increase the yield to some extent. The yield of glucan in this study was 7.4% of the weight of compressed yeast. The exact reason for the apparent differences in the yield of this product may be due to repetition of some steps and certain modifications such as, employing buchner funnel fitted with 0.45 µm Whatman filter paper during filtration by suction.

In this study, after estimating the melting point of the isolated glucan as 131°C, chemical analysis was carried out for estimating the total carbohydrate contents. The carbohydrate contents were estimated as 240 mg/ml. The upward mutorotation during hydrolysis indicated a β-configuration of glucose units in the polysaccharidic glucan.

The potential toxicity i.e. the minimal lethal dose of glucan was evaluated by estimating the LD 50 doses. Such experiments were considered essential before making any efforts to use glucan as a possible immunoadjuvant in vaccination studies. Several groups of mice were administered with a single intraperitoneal inoculation of glucan doses in an increasing logarithmic ratio (eg. successively doubled). In our study, the data, mortality graph and animal titration results clearly indicate that for a mice LD 50 dose was greater than 2 g/kg when given intraperitoneally.

The immunological studies relating to glucan were also carried out. The studies carried out to assess the adjuvanticity of isolated glucan, as per a predetermined immunization schedule. The antibody titers were evaluated by IHA and were found to be highest with 40 mg/kg dose.

The results obtained from this study will be helpful in our attempts to standardize the glucan preparation, for obtaining adjuvant samples with uniform immunobiological activity. In vaccination studies, eliminating the problem of glucan associated biological variance is of paramount importance. In the absence of such data any further use of glucan in vaccination experiments would be an unwise step. The need for obtaining consistently uniform results in any future

attempts towards development of an effective vaccine against parasitic infections cannot be compromised at any cost. In the past, the practice of obtaining glucan from one or two laboratories in Europe and United States is no longer going to suffice the growing need of this very important adjuvant, which is being increasingly used in a large number of vaccination studies against various parasitic infections.

INTRODUCTION

INTRODUCTION

Immunization of man and other animals by artificial means is carried out for several distinct purposes. The main aim of vaccination, whose achievement has already produced enormous benefit to mankind, is prophylactic immunization against infectious diseases - so that long - lasting effective immunity results from controlled stimulation of the immune system. By administering a harmless vaccine, rather than allowing an uncontrolled stimulation by natural/clinical infection of the microbes or parasites, a susceptible host is encouraged to generate adequate immune responses for obtaining a temporary/ lasting immunity. A higher concentration of such protective antibodies maintained in the circulation help in fighting or overcoming a subclinical infection. Ideally, the effect of such a prophylactic immunization must be long lasting. For practical and economic reasons, prophylactic immunization needs to be obtained with a minimum number of administrations and preferably by a simple infection employing the least amount of antigen - compatible with efficient immunization. When non-living agents are used some special means, of which the use of adjuvants is the main example, are needed to ensure the fulfilment of this requirement.

A basic difficulty in any attempt to survey adjuvants is that this deceptively simple term has various meanings. An adjuvant substance was defined by early research workers as one which stimulated the production of increased levels of antibody against protein antigens. This simplistic connotation was changed when it was demonstrated that in addition, adjuvants could stimulate cell mediated (delayed type hypersensitivity) or allergic responses against protein antigens and autoimmune reactions against homologous or heterologous tissue antigens. Later, adjuvant substances have been shown to cause either an increase in the immune response (immunopotentiation), or conversely a decrease in the immune response (immunodepression). The latter effect diminishes the physiologic value of the term adjuvant from the Latin adiuvare (to help) and more frequently, these substances are being referred to as immunomodulating agents.

Accomodating all the above views and according to recent discussions (W.H.O. Tech. Rep. Series 595, 1976; Elderman R. 1980), the immunomodulating adjuvants can be defined as substances that are incorporated into or injected simultaneously/concomitantly with an antigen. Such substances help in potentiating non-specific immune responses. A safe immunomodulating agent, in addition to numerous other factors, must be able to control the harmful effects of an otherwise active

substance. An immunomodulating agent should be able to increase the immunogenic potency of vaccines which are poorly immunogenic, or of relative low molecular mass, or genetically manipulated antigens. An immunomodulating adjuvant must therefore conform to the following attributes:

A) It should not induce hypersensitivity reactions to host's own tissues or to itself.

B) It must not contain cross-reactive antigens with human tissues.

C) It should not be carcinogenic teratogenic or abortogenic.

D) It should not be contaminated with substances that might stimulate other immunological events not involved in the specific response.

E) It must be biodegradable in human body.

F) It must never produce harmful nodules or abscesses if administered intramuscularly (im) or subcutaneously.

G) It should not be unstable in the manufactured vaccine for at least a period of two years.

The kind of immunostimulation produced by adjuvants should be measured and judged in relation to the sort of use for which they are intended. Also, acute and chronic toxicity assay of new candidate immunological adjuvants should be

performed in controlled studies with attention to both the local and the systemic sites, following both single and multiple injections.

For being clinically useful, vaccines containing adjuvants must have a specified shelf-life, for retaining useful adjuvant activity during the period and without developing properties that would increase adverse reactions. Unfortunately, these are indeed harsh conditions to impose on the adjuvant researchers, who must be cognizant with the biological activities of acceptable adjuvants.

DIFFERENT TYPES OF ADJUVANTS:

An astonishing variety of substances has been shown to act as adjuvants, but those which have found a use in vaccines, or which have been major subjects of experimental investigation, have been elucidated and discussed here.

1. Aluminium Salts:

Aluminium salts have been used particularly to raise the immunogenicity of toxoids in the development of antitoxic antisera. The first application of alum as an adjuvant was reported by Glenny et al. (1926) who used diptheria toxiods precipitated with potassium alum $KAl(SO_4)_2.12H_2O$, filtered and emulsified with water, in experimental vaccination of

guinea pigs. The animals injected subcutaneously with the suspension of alum-toxoid precipitate developed immunity. The immunity generated following such a treatment was measured by the onset of negative Schick Test at a much higher rate than the controls vaccinated with the original toxoid solution. The enhancement of immune response to alum precipitated toxoids have a two-fold action:

1. Providing a large absorptive areas to absorb fairly soluble (free diffusable) proteins and limit their biodiffusion.
2. Providing a particular attraction to immunogen-processing (phagocytic) cells <u>in vivo</u> by virtue of their low solubility (hydroxide).

Another important property of the aluminium salts is that they are not very toxic to cells mounting the immune response, unlike many other metal ions, such as Cu^2 which also aggregates or precipitates protein antigens.

Alum-precipitated vaccines were widely used for immunization of man and animals. As an alternative to alum precipitation, the toxoids are sometimes mixed with preformed gels of $Al(OH)_3$ or $AlPO_4$, to which they bind by ionic interaction to produce adsorbed vaccines. Aluminium salts thus remains the sole adjuvants acceptable for human vaccines at the present time.

2. Silica

The adjuvant effect of silica dust consisting of tridymite, a pseudohexagonal orthorombic form of crystalline SiO_2, injected intravenously into rabbits and rats was studied following subsequent antibody response of experimental animals to ovalbumin by Pernis and Paronetto (1962). The time interval between the administration of adjuvant and that of antigen was varied from 0-90 days. The enhancement of the immune response measured by the serum antibody levels and the number of antibody containing cells in lymphoid organs progressively increased with the time interval and reached to a maximum at 90 days. This is in marked contrast to the time relationship found in immunizations employing other kinds of adjuvants where the strongest response usually follows a simultaneous application of antigen and adjuvant. The silica treated rabbits exhibited a progressive enlargement of spleen and lymph nodes upto 10 times the normal size. Thus the injected silica dust induces extensive proliferation of lymphoid cells. Pernis & Paronetto (1962) pointed out that antigens adsorbed onto inert particles such as silicates, are likely to be ingested by phagocytic cells and provoke a foreign body-type macrophage response. Slow release of antigen from the local granuloma might allow increased stimulation of immunologically competent cells in the regional lymph nodes.

3. Freund's Adjuvants

Freund's adjuvants induce powerful cell-mediated responses, humoral immunity, break tolerance and potentiate tumour rejection. Their nomenclature honours the work of Jules Freund and his co-workers who established the conditions required to obtain reproducible activity, despite relying on materials of uncertain composition and of varying physical and chemical properties.

The history of these adjuvants is briefly outlined by Freund (1942, 1945). Towards the beginning of this century, it was found that an antigen injected into a tuberculous 'focus' gave heightened antibody titres and elicited a greater degree of delayed hypersensitivity in guinea pigs than when it was injected at other (non tuberculous) sites. Further more, they showed that heat-killed tubercle bacilli gave a marked tissue reaction, and antigens injected into this area of inflammation also elicited hypersensitivity. The same antigen injected into acute inflammatory foci produced with non-bacterial irritants including silica, tapioca or turpentine, however, did not elicit any greater immune response than upon injection into non-inflammatory tissue. This finding led Freund and Mc Dermott (1942) to develop adjuvants containing mycobacteria. A key constituent of their adjuvant formulation was a lanolin like substance contained in Aquaphor, which is

derived from wool fat and which contains added petrolatum hydrocarbons.

Further research has shown that adjuvant activity for induction of cell mediated immunity can be obtained also with variations from the classical (complete) Freund's adjuvant which consists of dead Mycobacterium suspended in mineral oil with an added emulsifier. Such variations can be achieved by (1) using either live or dead bacteria without an oily vehicle or, (2) by using an oily vehicle without a bacterial component.

The first variant has been studied with a view to using adjuvant immunotherapy to treat cancer, with viable Bacillus Calmette-Guerin (BCG), a strain of Mycobacterium bovis (Mackaness et al. 1973) given in saline as the sole adjuvant material.

The second variant seems to apply only to the immunization of rats, particularly in the context of sensitizing an animal to develop autointolerance to its own myelinated nervous tissue (allergic encephalomyelitis). For this purpose, the animals are usually challenged with a crude extract of spinal cord as the encephalitogen, a material which may contain natural emulsifiers with considerable lipids and other intrinsic pro-adjuvant components. As an extrinsic adjuvant it is only

necessary to use an oily vehicle which need not be a mineral oil (White, 1973). This phenomenon clearly contradicts the generally accepted view that it is the bacterial component which provides the essential adjuvanticity for cell mediated immunity.

This second varient has been evaluated at some length, for it has not always been appreciated that the vehicle may be of prime importance in determining the ultimate magnitude of an auto-allergic response. It has long been known that mineral oil (with or without an emulsifying agent) is an adequate adjuvant to stimulate a humoral response to simultaneously applied immunogen, and when used for this purpose it is commonly described as Freund's incomplete adjuvant.

Freund's incomplete adjuvant (i.e. oil emulsion adjuvants) is used in veterinary vaccines, but because of its tendency to cause sterile abscesses at the site of injection it has been abandoned for human use.

Freund's complete adjuvant (killed mycobacteria suspended in the oil) is too inflammatory even for veterinary vaccines, but is most effective for raising high titre antibodies in experimental animals.

4. Bacterial Lipopolysaccharides (Endotoxins)

These are toxic principles, usually designated as Lipopolysaccharides (LPS), produced by gram-negative bacteria. They are bound firmly to the bacterial cell wall in a complex with protein, from which they are shed when the cell undergoes lysis. The term 'endotoxin' is therefore a misnomer because neither these substances originate from outside the cells, nor they are always toxic. However, they do show the property of increasing resistance to infection. Lipopolysaccharides (LPS) when administered by various routes in microgram amounts to laboratory animals have been shown to elicit early IgM antibody responses (and later IgG responses in some species) against the polysaccharide components of the lipopolysaccharides.

The amount of antibody is much greater than that elicited by corresponding amounts of the polysaccharide component alone. When some other antigens are administered in small amounts simultaneously with lipopolysaccharides, the antibody response against them is also greatly increased. Lipopolysaccharides may therefore be regarded as adjuvants. They also have a wide variety of biological activities that are not directly related to their effect on the immune response. These include pyrogenicity, activation of plasminogen, adrenal

corticol stimulation, adherence of polymorphs and platelets to vascular endothelium (followed by leucocytosis). They further participate in the stimulation of acute phase protein synthesis (including properdin) by the liver, interferon release and stimulation and activation of the alternate complement pathway. Lipopolysaccharide is thought to act on a variety of different cells of which polymorphs and macrophages may be the most important, since these are the probable source of secondarily active factors(eg. interferon, lysosomal enzymes, plasminogen activator and other neutral proteases and possibly lymphocyte - activating factors).

The biological activities are wholly attributable to the presence of lipid A, which is common to all Lipopoly-saccharides. Lipid A is composed of two molecules of β (1-6)-linked glucosamine phosphate, to the amino group of which are attached myristic acid. Additional molecules of lauric and palmitic acid are attached at positions 3 and 4 to one of the glucosamine molecules. Modification of this structure by chemical means is possible. Pure lipid A aggregates to a viable extent in the presence of divalent cations and can only be reliably studied in monomeric form (conveniently as a trimethylamine salt prepared by electro-dialysis) or as a complex with an inert protein, such as bovine serum albumin.

Pure lipid A is pyrogenic in primates at a dose of 0.01 µg per kg of body weight, but very much larger quantities can be administered without proving fatal. Tolerance to the pyrogenic action is rapidly produced by repeated administration.

Although its wide spectrum of biological activities makes it unlikely that lipid A would normally be useful as an adjuvant, it is of interest because it has been shown to be a potent activator of a subset of B-lymphocytes (detected in mouse, rabbit or human spleen, but with a variable distribution in other tissues). Basically, it helps B lymphocytes to differentiate, divide and secrete IgM antibodies. This results in non-specific activation of the susceptible B-lymphocytes, irrespective of their antibody specificity. Infact, it increases the proliferation of B-lymphocytes already specifically stimulated by a separate antigen administered simultaneously.

5. Calmette-Guerin Bacillus (B.C.G.)

B.C.G. a potent immunostimulant is presently the most commonly used non-specific stimulating substance in human therapy. As an immunostimulant BCG increases resistance in mice to several unrelated bacterial infections and markedly enhances the clearance of particulate materials by the reticuloendothelial system. Such stimulation persists for a very

long period. The BCG enhances the production of antibodies and increases the number of spleen antibody producing cells. Also it increases delayed type hypersensitivity for particulate and soluble antigens, accelerates the rejection of skin allografts and transplanted tumours in mice and inhibits carcinogenesis. It also tends to modify the action of chemotherapeutic drugs such as cyclophosphamide (Mackaness, 1970). In respect to toxicity the BCG may act synergistically or antagonistically with certain chemotherapeutic drugs.

The BCG could help to promote the immunological rejection of tumours or augment resistance to infection in two ways. The immune rejection of tumour depends on the potentiating effect that mycobacteria exert on the immune response in general. The augmentation of resistance largely depends on inducing reticuloendothelial hyperplasia and hyperactivity. This is seen not only in animals injected with BCG but also when subjects with cell-mediated immunity are deliberately or inadvertently exposed to the specific antigen. The first effect of BCG also serves to enhance the hosts capacity to make a specifically directed attack on the tumour cells or the parasite. The second attribute helps in the final expression of resistance by creating a more efficient scavenging system. The latter seems at present to be as crucial in promoting anti-tumour immunity as in augmenting resistance to some infectious diseases (Mackaness, 1962).

Stimulation of cell-mediated immunity is best achieved with a freshly prepared culture of BCG containing a high proportion of live bacilli. The mode of preparation and preservation on bacterial suspensions and the strains used are of importance for the efficiency of BCG adjuvanticity. There is evidence that optimum activity depends upon the use of bacteria from the pellicle growth rather than from submerged culture. Killed bacilli act mainly to increase circulating antibodies.

In order to avoid any possible hazards arising from the use of strains of live, though attenuated bacilli in human subjects, efforts have been made to substitute saprophytic bacteria or to use subcellular fractions of mycobacteria.

6. Polynucleotides and Nucleic Acids

Much of the earlier literature on this subject has been reviewed in a book (Beers & Braun, 1971). Originally, interest was aroused in these polyphosphates which contain the common purines and pyrimidines, since it was believed that endogenous oligonucleotides formed in some quantity - after X-irradiation or treatment with cytostatic drugs or cell necrotic agents such as endotoxins - were responsible

for the heightened (humoral) immune responsiveness observed in all these conditions. Concurrent discoveries that these substances also induce immunity further helped in generating research interest in them.

Polynucleotides are formed following the action of enzyme, polynucleotide phosphorylase, isolated from Streptomyces lysodeikticus on the desired mononucleotide diphosphates. When the polymerized single strands from opposite base pairs are mixed, a double helix is formed. Thus polyadenylic acid mixed with its opposite base pair, polyuridylic acid, forms the poly A:U complex. Similarly polyinosinic acid mixed with polycytidylic acid forms poly I:C complex.

The potential usefulness of such complexes as immune stimulants was suggested initially by the finding of Jaraslow & Taliaferro (1956) who indicated that nucleic acid rich tissues have a restorative effect in animals rendered deficient in antibody forming capacity following irradiation. Subsequently, heterologous and homologous DNA and RNA, as well as the low molecular weight products, resulting from nuclease treatment were shown to act as adjuvants in the normal immune response (Merritt & Johnson, 1965). Following advances in the field of nucleic acid synthesis, the synthetic polynucleotides became available and these likewise, were found as effective adjuvants (Johnson, 1976).

The polynucleotides have been found to enhance antibody response to many antigens in a variety of mammalian and avian species. Their efficacy as adjuvants is illustrated by their capacity to render proteins in low concentrations of 10-1 ng antigenic. They are also found to be effective by multiple routes and need not be given together with antigens (Johnson, 1976).

Polynucleotides can affect host defence systems, non-specifically, against a wide spectrum of infectious agents. Irrespective of the mechanisms the potentiation of immunologic vaccines by polynucleotides offers promise for the future. This is particularly so for certain viral vaccines, since this adjuvant's ability to render immunogenic antigen concentrations in nanograms concentrations may abrogate the need for amounts of purified virus material difficult to achieve.

7. Muramyl Dipeptide (MDP)

Approximately ten years ago, synthesis of N-acetyl-muramyl-L-alanyl-D-isoglutamine (muramyl dipeptide or MDP), representing the copy of a peptidoglycan fragment present in bacterial cell walls was achieved by Lederer (1974) and Kotani (1975). Development of this new agent which is capable of potentiating the antibody production without many side-effects is, therefore, being actively pursued. Several

hundred synthetic muramyl peptide derivatives or analogues are produced in laboratories, allowing a good understanding of the relationship between chemical structure and biological activities of these molecules (Lederer, 1980). As in the case of many immunostimulating agents, studies have shown that MDP may possess a wide spectrum of properties besides their manifest immunological capacity. These molecules also have general pharmacological effects including those which can be considered as neuropharmacological (Chedid, 1983).

MDP in the smallest structure capable of including all the adjuvant activities produced by Mycobacteria in a water-in-oil-emulsion. However, since this powerful combination cannot be used, MDP could only be administered to humans in aqueous media. Under these conditions the adjuvant has been shown to augment the humoral antibody response to several antigens, although it has been shown to produce certain side effects such as fever (Chedid, 1983). By chemical modification, it has been possible to eliminate these unwanted properties and to retain its immunopotentiating activity. Certain lipophilic derivates of MDP have also been shown to increase the cell mediated immune response even when administered in saline (Carelli et al., 1981). They, however, also seem to produce stronger side effects than most of their hydrophilic congeneres and presently have not reached the stage of

clinical trials. The antibody class that an adjuvant can turn on is of utmost importance in view of conferring 'real' immunity, yet paradoxically most studies do not emphasize this aspect and generally focus on producing probably unnecessary high antibody titres.

MDP and adjuvant active derivatives can thus enhance both specific and non-specific immunity. Moreover various constructs containing MDP could also induce a broader spectrum of antiviral, non-specific immunity in addition to the protection due to specific antibodies. Muramyl peptides may also constitute very favourable tools for a better understanding of the relationship between the immune and the central nervous system. They can therefore represent potential drugs in the field of veterinary and human medicine.

8. Glucan:

Glucan, an extract from the inner cell wall of Saccharomyces cerevisae, has been variously claimed as a non-specific stimulant of the reticuloendothelial system. Beta 1,3 linked glucans have been demonstrated in a variety of laboratories to significantly enhance humoral and cellular immunity. Glucans have been uniformly demonstrated to materially modify the infectibility of experimental animals

to a wide variety of agents such as bacteria, fungus, virus and parasites. Additionally, glucans have been demonstrated to significantly alter growth and development of either syngenic, allogenic and autochthomous tumours in experimental animals as also in a variety of human malignancies. They also have, through their ability to modify macrophage function, a profound influence on hemopoietic activity of bone marrow and spleen. In numerous studies conducted to date, glucans have also been demonstrated to effectively function as an adjuvant for tumour, viral, bacterial and parasitic vaccines. The broad spectrum of immunopharmacological activity of glucans, coupled with the end metabolite being glucose, projects the continued development of these polyglucose immunomodulators for therapeutic purposes against a variety of infectious and neoplastic diseases, as well as their development as effective, non-toxic adjuvants. At the moment, the broad spectrum immunopharmacological activities have helped glucan appear as an effective adjuvant. The knowledge in this field has been comprehensively reviewed and discussed in further part of this thesis.

THE POSSIBLE MODE OF ACTION OF ADJUVANTS ON THE IMMUNE RESPONSE

Some adjuvants exert their effects on a wide range of immune responses to an antigen, but in other cases the

effects are more or less selective, influencing certain types of response rather than others. This could have practical application : it is likely, for example that serum antibodies play a major role in resistance against certain groups of viruses, eg. enteroviruses, whereas cell mediated immunity makes an important contribution to host resistance against other viruses. If it was possible by the judicious use of adjuvants or antigen manipulation to increase the humoral immune response elicited by some virus vaccines and the cell mediated immune response elicited by others, a considerable advance could have been made. Any procedure that increases antibody formation rather than cell mediated immunity might facilitate the retention of renal allografts, whereas the reverse might favour the rejection of tumours. Atopic patients would benefit if formation of other classes of antibodies, instead of cell-bound sensitizing antibodies, could be increased by exposure to antigens in the presence of suitable adjuvants.

Adjuvants can be employed as probes for analyzing the subtle cellular interactions which occur during immune responses. Antigen doses which in the absence of adjuvants induce specific immunological unresponsiveness elicit antibody formation in the presence of adjuvants with protein antigens. Incomplete

Freund's adjuvant usually stimulates antibody formation and this can block subsequent attempts to induce delayed hypersensitivity against the same antigen. Protein antigens administered in Freund's complete adjuvant often elicit cell-mediated immunity, affecting the subclass of antibody formed. Possible effects of adjuvants on other systems may also be of biological importance, including effects on phagocytic cells and on the effector cells involved in antibody formation and cell mediated immunity.

Eventually, the effects of various adjuvants will be defined in terms of whether they stimulate the formation of particular class or subclass of antibody, or cell mediated immunity. It is already clear that all adjuvants do not work in the same way. In order to analyze the mode of action of adjuvants, it is essential to know on which cell types in the immune response they exert their effects, directly or indirectly.

ADJUVANT ACTION AT THE CELLULAR LEVEL

It has become clear during the last decade that the immune response usually depends on a complex interaction between antigen and one or more of several cell types. The possibility that contact with antigen can elicit in various lymphocyte categories one or the other type of the two

mutually exclusive immunological states (immunity and tolerance). It is envisaged that adjuvants can act on any one or more of the cell types and where operational immunopotentiation is observed, there may be stimulation of different combinations of these cell types.

Various substances with empirically defined adjuvant properties may stimulate macrophages with reference to any one of their several roles in immune responses. These include:

(1) The production of processed antigen with increased immunogenicity.

(2) The removal of excess antigen, thereby protecting the animal against tolerance induction.

(3) Improved and/or prolonged presentation of lymphocytes of antigen held on their surface membrane.

(4) Action as cellular reservoirs of antigen that can be released over an extended period (it is implicit here that repeated stimulation by antigen over an extended period will produce higher antibody titres than a single exposure).

(5) The synthesis and release of (non-specific) lymphocytes-activating and/or inhibiting factor.

Many adjuvants and immune complexes potentiate the trapping of circulatory lymphocytes in draining lymphoid organs. This mechanism, which may be mediated by stimulation of certain specialized macrophages (littoral cells), increases the probability of successful contact between all the necessary cellular protagonists in the immune response.

There are a few reasonably well defined model systems where an important aspect of adjuvant action is to switch cells from a pathway leading towards tolerance to one leading to immune differentiations. Both adjuvant action and tolerance induction can be at the level of any one or any combination of the lymphocyte subtypes such as T (helper), T (suppressor), T (killer), B (μ class) and B (γ class).

Cell differentiation is a time dependent phenomenon and it is unequivocal that at least some elements of its mechanisms are involved in immune differentiation. Consequently, it is of interest that the stimulatory and suppressive effects of both adjuvants and immunosuppressive procedures are dependent on the timing of their administration (and their doses) in relation to that of antigen. A theoretically possible mechanism for certain adjuvants that awaits exploration is that they act as selective suppressors of the regulatory

T (suppressor) - cells that are normally elicited by an administered antigen. Regulatory mechanisms involving B-cell products (antibody), which are stimulated by Freund's adjuvant have been demonstrated in Chickens (White, 1973).

In brief, it is envisaged that adjuvants can act at the cellular level in one or more of the following three ways:-

1) By helping lymphocytes to 'decide' to enter a pathway of immune rather than tolerance differentiation.

2) By amplifying 'post-decision' proliferation of immune induced lymphocytes, either directly or via the release of stimulatory factors from macrophages, thereby increasing the size of the eventual clones of antibody producing cells.

3) By increasing the efficiency of macrophages for storage and preservation of antigen to the extent that the specific stimulus is prolonged, thereby encouraging the 'post-decision' proliferation in a different way.

This last point serves to emphasize that intermediary macrophages may be an important factor in many - if not most - examples of immune induction.

ADJUVANTS IN ACTIVATION OF MACROPHAGES

The access of foreign particles to the tissues is followed by their engulfment by both microphages (granulocytes) and macrophages.

Since the latter cells are far more persistent than granulocytes in the reactions at the site of a continuing infection or invasion by allogeneic or syngeneic cells (neoplasm or normal allograft), it commands much greater attention from the immunologists. A local inflammatory reaction generated by an adjuvant mainly arises from invading blood monocytes, although local multiplication of tissue macrophages can also occur. The bulk of ingested materials, including bacteria are rapidly broken down in the polylysosomes, though significant amounts of materials derived from them may be retained in macromolecular form at the macrophage surface where they function as potent immunogens. The macrophages obtained from recently injected animals exhibit enhanced ability to kill and digest such ingested bacilli. The various cellular adaptations involved are referred to as 'activation' and entail the synthesis of large quantities of lysosomal and other enzymes, with a variety of end results.

EFFECT OF ADJUVANTS ON ANTIBODY CLASSES

Many adjuvants induce a preferential synthesis of one or more antibody classes. The triggering of IgE antibody class is particularly dependent on the type of adjuvant used to stimulate the primary response. Both B. pertussis and particles of $Al(OH)_3$ on which antigen is adsorbed are very efficient adjuvants for enhancing the synthesis of IgE antibody in rabbits and rodents. On the other hand, Freund's complete adjuvant favours the synthesis of IgG antibody in most species. Mice immunized with a combination of thymus-dependent antigen such as human serum albumin and B. pertussis produced more IgG_1 and IgG_{2b} than IgG_{2a}. Guinea pigs immunized with Freund's complete adjuvant produced IgG_1 and IgG_2 antibodies, whereas when the animals stimulated with Freund's incomplete adjuvant produce almost entirely IgG_1 antibody. Intraperitoneal immunization of rabbits with alum-precipitated antigen enhances the production of IgE antibody, whereas immunization with Freund's complete adjuvant enhances IgG.

EFFECT OF ADJUVANTS ON ANTIBODY AFFINITY

It has been reported that small amounts of antigen acting over a prolonged period of time (as in the case of

Freund's complete adjuvant) stimulate the production of antibodies against any given determinant whose average affinity increases with time. When the antigen is a complex molecule possessing a variety of determinants, the antibodies become more avid as measured, for example, by the capacity of an antiserum to form indissociable complexes with the antigen. Increase in avidity is explained in part by the production of antibodies against more determinants as stimulation is prolonged. The changes in antibody affinity occur in antibodies of the IgG class and only to a minor extent in those of the IgM class.

THE DEPOT HYPOTHESIS

This states that the adjuvant immobilizes the antigen, preventing its dissemination from the site of injection, or the draining lymph node and causing it to be slowly released into the immune system. A slow release of antigen causes prolonged antigenic stimulation, and a secondary-type antibody response can likely develop. Studies with radiolabelled proteins have confirmed that the inorganic gels and emulsions do retain the antigen. Also, for both of these adjuvants, a good physical association with the antigen is necessary for the full adjuvant activity. The immunogenicity of proteins which do not adsorb to aluminium salts is poorly enhanced by this

adjuvant and oil-in-water emulsions are not nearly as effective as water-in-oil.

The depot hypothesis has been challanged on two grounds. First, the excision of the antigen adjuvant depot from a subcutaneous site soon after infection does not prevent boosting of the immune response. However, these experiments do not completely refute the hypothesis because it is known that complexes of antigen and adjuvant are very rapidly distributed into the draining lymph node, and may gradually release their payload, in these tissues. The only conclusion that can be drawn is that the continuing presence of the subcutaneous depot which may, if left undisturbed remains for weeks or months, is not required. Secondly, there are some antigens which are susceptible to the adjuvant action of gels or emulsions do not provoke any antibody response when injected on their own in multiple, small doses for obtaining a slow release. This indicates that slow release may not by itself be sufficient to account for adjuvant activity, although it may still be a contributory factor in combination with effects of the adjuvant on the immune system.

FACTORS IN ADJUVANT EFFECTS

Although much remains to be learned about mechanisms

underlying adjuvant action, it is already clear that adjuvants exert their effects in several more or less distinct ways.

1) Slow Release

Slowing the release of labelled antigen injected subcutaneously in water-in-oil emulsion has a half life of 14 days, while free antigen or alum-precipitated antigen has a half life of less than one day, the difference in blood levels is however much less marked (Talmage & Dixon, 1953). However, antibody response to soluble antigens such as BSA or bovine gamma-globulin can be increased by injecting adjuvants at different sites from those of antigen injection (Dresser, 1968).

Endotoxin was not found to have any effect on the trapping, retention and localization of labelled serum albumin in lymph nodes (Ada et al., 1968). All these results make it unlikely that the depot effect is more than a contributory factor to the mode of action of some adjuvants.

2. Antigen Denaturation

Certain antigens can be denatured or made particulate by emulsification or adsorption onto alum, bentonite or other particles. It is well known that denatured serum proteins,

especially gammaglobulin, tend to be immunogenic, whereas the native proteins are tolerogenic (Dresser & Mitchison, 1968). Denatured or particulate antigen readily becomes associated with membranes of macrophages and other immunocompetent cells (eg. dendritiz cell of lymph node and spleen). This may facilitate the presentation of antigen to lymphocytes in a highly immunogenic form.

3. Recruitment of Reactive Cells:

Histologically, the site of inoculation of Freund's adjuvant becomes a granuloma, with infiltration of macrophages, lymphocytes and other cells. These cells are exposed to a relatively high concentration of antigen locally and in the draining lymph node.

4. Stimulation of the Proliferation and Differentiation of Immunocompetent Cells

The fact that the lipopolysaccharide is a selective B cell mitogen and can exert adjuvant effect through B cells is unlikely to be a coincidence. Taub et al. (1970) have reported that vitamin A, which is an efficient adjuvant but not immunogenic, induces blast transformation and proliferation of cells in the thymus dependent areas of draining lymph nodes, whereas other substances, such as paraffin oil, without adjuvant

activity have at most fewer effects on the thymus - dependent areas of lymph nodes. The allogenic effect is recognised to provide a general stimulus to the proliferation of lymphocytes. Hyperplasia of the reticuloendothelial system and the proliferation of lymphoid cells in animals treated with adjuvants are well known. The combined stimulation of clones of lymphocytes by antigen and adjuvant is likely to be greater than that achieved single handedly. However, in adjuvant-treated animals there is an overall increase in synthesis of immunoglobulin, not just of specific antibody (Binaghi, 1966). The same author found that although Freund's adjuvant in guinea pigs causes the production of more γ^2 and γ^1 antibody against the injected antigen, the percentage rise in serum γ^2 globulin helps other immunocompetent cells to get stimulated by the adjuvant.

5) Cellular stimulation

In addition to antibody formation, some adjuvants stimulate cell-mediated immunity. The classical Freund's complete adjuvant facilitates the development of delayed hypersensitivity against serum protein antigens, in contrast to the incomplete adjuvant which stimulates antibody formation but not delayed hypersensitivity. This switch mechanism may be important, especially in relation to tumour immunity. Thus, it was found

that Freund's complete adjuvant diminishes the number of tumors appearing after injection of adenovirus type 12 in hamsters (Berman et al., 1967). The type of adjuvant timing and route of administration and other factors greatly influence the end result.

6) Antibody Dependent Cellular Immunity

A new factor to be taken into consideration is the effect of adjuvants on antibody-dependent cell-mediated immunity. The number of effector cells is increased by Freund's complete adjuvant, the effects of other adjuvants still remains to be investigated. This system could be involved in immunity against certain tumours.

HISTORICAL BACKGROUND RELATIVE TO THE DEVELOPMENT OF GLUCANS

In early fifties Prof. N.R. DiLuzio of the Tulane University in New Orleans and his colleagues in their laboratory attempted to define the role of macrophages in the metabolism of chylomicrons and cholesterol and their possible role in the development of coronary artery disease. At that time the technique employed was to depress the macrophages and ascertain the consequence using colloidal carbon or trypan blue for producing the so called 'reticuloendothelial blockade'. The accumulation of dyes and particulates in macrophage elements might lead to the assumption that these

cells are functionally impaired, functional phagocytic data indicated marked activation of these cells. Thus the technique of reticuloendothelial blockade was found to be extremely inappropriate in the evaluation of the contribution of macrophages to homeostasis.

In 1957 Benacerof and Sebestyn presented a report on the influence of intravenously administered Zymosan, a crude S. cerevisae cell wall fraction on macrophage activation. One of the findings of this study suggested that there occurs a stimulation of macrophages as reflected by phagocytic activity, as well as the development of hepatosplenomegaly.

Employing this initial observation, an attempt was made to isolate the active component in zymosan, which related to its ability to enhance macrophage function as well as induce proliferation of macrophage elements in the liver, lung and spleen. The results of the above study published in 1961 (Riggi & DiLuzio, 1961) indicated that the active fraction of Zymosan was Glucan. In fact it was this study which ushered, the era of glucan research. Retrospectively, the results of such researches over a quarter of a century has contributed a lot towards the identification of Glucan as an effective immunomodulator for therapeutic purposes.

Chihara et al. in 1969 subsequently isolated β-1-6, β-1,3 glucan from Lentinus edodes which was later designated as lentinan. They also demonstrated lentinan's influence on the inhibition of Sarcoma 180 in allogenic mice. Since that time a variety of glucan(s) have been prepared from an extensive assay of single and multicellular fungi. The role of these compounds in enhancing the host resistance to either infections or malignancies has now been thoroughly studied. In general, most effective immunomodulating polysaccharides of the glucan class have been derived from S. cerevisiae, L. edodes and S. commune.

ADJUVANT ACTIVITY OF GLUCAN

In 1963 Wooles and Di Luzio demonstrated in a murine model that the administration of glucan is associated with an increased vascular clearance of sheep red cells and a resulting enhancement of both the primary and secondary immune response. Conversely, the administration of methyl palmitate which reduces phagocytic events was associated with a profound depression of both primary and secondary hemolysin titers. These observations of the pivotal role of macrophages in the induction of immunity have since been confirmed in different laboratories. These studies clearly indicated that it is possible to control the fate of particulate antigens and to modulate immune responsiveness by agents which influence macrophage behaviour.

The adjuvant activity of glucan as demonstrated by Holbrook et al. (1981) further confirmed that glucan upon simultaneous inoculation with formalin killed erythrocytic stages of Plasmodium berghei induces enhanced degree of resistance in mice against subsequent infections with variable parasites. The injections of formalin killed erythrocytic stages of P. berghei alone did not produced a similar effect. The mice immunized with glucan and dead parasite preparations showed 100% survival following challenge. In contrast, only 28% of the mice receiving antigen alone survived. These studies on glucan and antigen parasites were extended by this group to evaluate the effects of Leishmania donovani infections using glucan as an adjuvant (Cook&Holbrook, 1982; Cook & Holbrook, 1983; Holbrook & Cook, 1983). Mice were immunized by a series of intravenous infections with formalin-killed L. donovani promastigotes alone, or in combination with glucan. The mice which were immunized with dead parasites and glucan exhibited resistance against challenge upto 80 days, while the injection of promastigotes alone conferred no measurable resistance against infection. Moreover they have also reported that intravenous, intraperitoneal or subcutaneous administration of formalin killed L. donovani promastigotes with glucan would confer a significant degree of protection against subsequent infection with variable promastigotes. Intramuscular

immunization was not effective.

Additional studies showed the unique adjuvant activity of glucan (Ahmad et al. 1980) where a 100% protection was obtained against Entamoeba histolytica infections. In these studies glucan was employed as an adjuvant with E.histolytica extracts. The glucan-antigen immunized guinea pigs were devoid of ulcerative amebic lesions which were so prominent in the control groups. It has been reported that glucan is an effective adjuvant in enhancing immunity to Babesia microti infections (Benach et al., 1982).

Reynolds et al. (1980) also demonstrated that mice given glucan combined with marginally immunogenic doses of venezuelan equine encephalomyelitis vaccine were more resistant to virus challenges than mice given Freund's complete adjuvant (FCA) plus vaccine, or vaccine alone. Indeed in all these studies conducted to date, glucan appears to be more effective and less toxic adjuvant than FCA. The humoral immunity evaluated in cynomologus monkeys following administration of glucan with Venezuelan equine encephalomyelitis vaccine was significantly enhanced in contrast to vaccine controls. These studies denote that glucan is a very effective adjuvant for the induction of humoral and protective immunity to certain viruses.

The enhancement of anti-viral host defense mechanisms by glucan was further extended through observations made by Williams and Di Luzio (1980) on the modification of murine viral hepatitis (MHV). In these studies it was observed that maximum effectiveness in modifying survival to lethal MHV challenge resulted when glucan was administered prior to, as well as after, viral challenge. In contrast to the profound hepatic parenchymal cell necrosis observed in control mice, glucan-treated mice exhibited very limited pathology. The maintenance of hepatic structure was associated with minimal alterations in plasma enzymes such as SGOT and SGPT which reflect the structural integrity of hepatic parenchymal cells. Like wise BSP clearance, which was markedly impaired in control mice with hepatitis, was essentially normal in the glucan-treated viral infected group. Additionally, control mice challenged with MHV A-59 hepatitis showed significant impairement in macrophage phagocytic function. In contrast, pretreatment with glucan prevented macrophage impairment and resulted in a hyperphagocytic state in virus challenged mice. These studies along with those on E. coli suggest that maintenance of an activated state of phagocytic function, as well as other possible macrophage secretory and/or metabolic activities, resulted in increased survival and the inhibition of cellular injury. Their composite findings denote that macrophage

stimulants such as glucan may play a significant role in the modification of viral - induced lesions.

Extensive studies have also been done (Williams et al., 1983) to evaluate the influence of intraperitoneal inoculation of glucan in E. coli induced experimental peritonitis and sepsis. In these studies glucan was administered prior to i.p. challenge with 1.0×10^8 E. coli. Data in these experiments indicated an approximately 97% survival in the glucan treated group, in contrast to an approximated 19% survival in the control group. The difference in degree of sepsis between the control and experimental groups was pronounced. The control group showed exponential increases in the blood level of E. coli throughout the 24 hr period of study with mean level of 2×10^7/ml in the blood. In contrast, the glucan-treated groups, which had identical blood levels of E. coli at early intervals, were almost sterile at later intervals.

USE OF GLUCAN IN THE DEVELOPMENT OF VACCINE

Rapid advances made during the last few years in the development of adjuvants have led many to believe that adjuvants of practical and commercial value would soon emerge out. These hopes however have not yet been realized because some

crucial problems of adverse side effects still remain to be solved. There may be possible problems such as:

(1) Sensitization to the agent itself or to the common antigens between agents and the host or auto-antigens.

(2) Increased formation of blocking antibodies of suppressor cells.

(3) Toxicity for myelolymphoid cells or neoplastic transformation of these cells.

Infections can be controlled by direct chemotherapy or by agents capable of increasing the specific or non-specific immune responses of the host. At the present time vaccination is the oldest and most successful achievement of clinical immunology. Still there exists many experimental models showing that it is possible to increase natural resistance to infections by administrating bacterial products such as endotoxins or BCG (Mackaness et al., 1974). It is clear that the opportunity offered by modern biotechnology (monoclonal antibodies, genetic engineering, DNA sequencing, peptide synthesis, purification methods etc.) will be taken advantage of to produce improved vaccines. Indeed many antigens have already been prepared by genetic engineering (Chanock et al., 1984), including a hepatitis B surface antigen produced by yeast which is currently in clinical trials (Hilleman, 1985).

During the last four years a great deal of efforts have been devoted to the designing and production of synthetic antigens capable of evoking protective or neutralizing immune responses towards pathogens and hormones. These studies have supported the notion that to be immunogenic, a preparation has to contain three entities: the antigenic determinants which will evoke antibodies specific for the pathogen or hormone, a carrier molecule affording suitable presentation of these determinants to the immune system and an immunopotentiating moiety which will augment the response. These elements are all present in natural vaccines.

However natural vaccines also contain other structures, in most cases poorly defined, which are useless for vaccination and are even capable of inducing unwanted side effects. Thus one of the main reasons for developing vaccines is to obtain efficient preparations capable of producing high antibody titres.

Focussing on the role of carriers and of adjuvants, polysaccharide β-1,3 glucan is a good candidate for utilization in vaccine preparations. Glucan as a molecular entity is essentially non-toxic while it generates utilizable carbohydrates during metabolism.

The adjuvant activity of glucan in enhancing immunity

to protozoan infection has been demonstrated by the studies of Holbrook and Cook, 1983 and Ahmad et al. (1980) as described earlier.

In summary, extensive experiments which have been conducted in a variety of laboratories have indicated that β-1,3 glucans can be effectively employed in the modification of major microparasitic diseases and tumors. Furthermore, the broad spectrum of immunobiological activities of glucan against microparasitic diseases and tumors coupled with the observations that glucan can be employed as a very effective adjuvant for a variety of non-self entities including viruses, bacteria, fungi, parasites and tumor cells protends the development of glucans for prophylactic and therapeutic purposes in both clinical and veterinary medicine.

It is also projected that glucan research efforts will provide a significant contribution to the understanding of the processes of infection within various hosts and contribute to new and more effective vaccines, as well as drugs, which have the ability to interact synergistically with all current modes of therapy.

ISOLATION OF GLUCAN BY EARLIER WORKERS

Glucan, a neutral particulate β-1,3 polyglucose from

the cell wall of S. cerevisiae, was first isolated by Hassid *et al. in 1941. They took 2700 grams of compressed bakers* yeast grown on a grain mesh and allowed its digestion in two portions in 4 liter flasks. Each flask containing 2 liters yeast was reacted with 3% sodium hydroxide by heating on a boiling water bath for four hours. The dark brown alkaline digest was allowed to remain at room temperature for one day, the supernatant liquid was then decanted and 2 liters of fresh 3% sodium hydroxide was added to each flask. The flasks with the contents were shaken, placed for two hours on a boiling water bath and then allowed to cool overnight. The alkaline supernatant liquid, which again became dark brown was decanted, the residue acidified with about 800 cc of concentrated hydrochloric acid and 2 litres of 3% hydrochloric acid added to each flask. The flasks with the contents were digested for several hours on a water bath, then the supernatant liquid was cooled, decanted and digested with 3% hydrochloric acid. The final acid digest was decanted, the residue washed with distilled water and centrifuged, followed by resuspension in water and centrifugation. The residue was then washed well with boiling water, centrifuged and the combined residues from the two flasks suspended in 1 liter of alcohol and stored at room temperature for several days. The brownish-red alcohol solution was centrifuged off, the residue

(suspended in alcohol and then filtered by suction) was washed with ether and dried at 70°C in vaccum for four hours. It was allowed to dry into a horny hard brownish mass, then after grounding it to a fine powder in a ball mill a grayish white powder was obtained. The yield was 6.4%, since the moisture content of the compressed yeast averages around 70%.

A modification of the method of Hassid et al. (1941) was earlier reported by Di Luzio et al. (1979) to prepare particulate glucan from S. cerevisae . The Di Luzio's modified procedure is discussed briefly as follows:

Using a 6 liter flask 540 g of dry yeast was suspended in 3 litres of 3% aqueous sodium hydroxide solution. The suspension was placed in a boiling water bath for 4 hrs, cooled overnight and the supernatant decanted. This procedure was repeated three times. The residue was then acidified with 300 ml of concentrated hydrochloric acid plus 2 l of 3% hydrochloric acid and placed in a boiling water bath for 4 hrs. The suspension was allowed to stand over night and the supernatant decanted. The residue was further digested with 3 l of 3% hydrochloric acid at 100°C for 4 hrs, cooled overnight and decanted. The 3% hydrochloric acid digestion was repeated twice. The residue was then washed three times with distilled water. One litre of ethyl alcohol was added

to the residue, mixed thoroughly and allowed to stand for until 24 hrs for maximum extraction. The dark reddish brown alcohol supernatant was aspirated from the residue and discarded. The alcohol extraction procedure was repeated twice. The alcohol was removed by washing the residue with distilled water. The preparation was then collected by centrifugation. The residue was light brown in colour. The yield was 6.8% of the weight of dry yeast.

In 1973 Manners et al. also made an attempt to extract glucan from the cell-wall of baker's compressed yeast. About 2 kg yeast was first dispersed in 1.6 litres of 6% (w/v) NaOH and stirred at room temperature for 24 hours. Distilled water (12 litres) was then added and the insoluble material collected by centrifugation at 1200 g for 14 min. This material was suspended in 3 litres of 3% (w/v) NaOH and heated at 75°C for 3 hrs, then allowed to cool and the suspension was stirred for 16 hrs at room temperature.

After dilution with 3 litres of distilled water the insoluble material was again collected by centrifugation at 1200 g for 15 min. The residue was extracted again with hot 3% NaOH, adjusted to pH 4.5 (with HCl) and collected by centrifugation. The insoluble cell wall material was washed thrice with water, once with ethanol and twice with ether. The residue

was light brown in colour. On drying in air at 37°C, the yield was 47.9 g or 2.4% of the weight of compressed yeast.

In 1958 Peat et al. also isolated glucan from fresh baker's yeast. About 6 kg of fresh baker's yeast was dispersed in 5 liters of 6% (w/v) NaOH and stirred at 75°C for 24 hrs. About 14 litres distilled water was then added and insoluble material was collected by centrifugation at 1200 g for 15 min. This whole material was then suspended in 6 liters of 0.5 M acetic acid at 90°C for 3 hrs. After cooling, the supernatant was centrifuged. The residue was washed with water in a centrifuge, then suspended in 0.02 M sodium acetate (pH 7.0, 2 lit.) and heated for 1 hr at 135°C in an autoclave. After cooling, 2 lit. of water was added and the residue separated in a centrifuge. The supernatant gave an intense red colour with iodine, indicating the extraction of glycogen. After six washings of the residue with water (1.5 lit. each) the wash-liquid was achronic, but on further autoclaving at 135°C in 3 lit. of water extracted more glycogen. The residue was therefore centrifuged, washed three times with water (2 lit. each) and again autoclaved. The gelatinous solid was dehydrated with ethanol (3 vol.), centrifuged and washed successively with ethanol, ether and light petroleum. The product (39 grams) was a light buff-coloured powder. The total yield was 0.65% of the weight of fresh bakers yeast.

AIMS & OBJECTIVES

AIMS AND OBJECTIVES

The present study was aimed to isolate β-1,3 Glucan, a cell wall component from compressed bakers yeast Saccharomyces cerevisae. These investigations were also carried out to evaluate the toxicity of glucan in an experimental animal model and to further assess its adjuvanticity. Currently, β-1,3 Glucan is commonly employed as an immunomodulator in vaccination against a large number of parasitic infections. A detailed plan of the above study was as follows:

1) β-1,3 Glucan was isolated and purified from compressed bakers yeast (S. cerevisae) to get a good yield by employing different modifications in the procedures already described.

2) Chemical characterization of isolated β-1,3 Glucan.

3) Toxicity studies of the isolated glucan in mice and the determination of LD 50 values.

4) The immunological assays for determining Glucan's adjuvanticity were carried out by means of IHA tests for estimating the maximum antibody titer following immunization of test animals with different doses of glucan.

As a short term objective, the study was designed to obtain sufficiently large amounts of β-1,3 Glucan for further

use in vaccination studies - as also for its possible uses in any future attempt towards development of an effective vaccine against parasitic infections such as Amoebiasis, Leishmaniasis etc.

MATERIALS

MATERIALS

1. Animals

About 10-12 weeks old healthy mice weighing approximately 12-15 grams employed in this study were obtained from M/S Laboaids, Meerut. The rabbits employed in this study were also purchased from the same company.

2. Animal Food

Pellet diet for experimental animals was obtained from M/S Hindustan Lever Ltd., India.

3. Yeast (Saccharomyces cerevisae)

Compressed cakes of live bakers yeast, each weighing about 500 gms were obtained from M/S Bio-Food Pvt. Ltd., India.

4. Anticoagulant

3.8% sodium citrate was used as an anticoagulant.

5. Preservative

Sodium azide was purchased from Riedel, Germany.

6. Filter paper

Whatman filter paper of pore size 0.45 µm was obtained from International Traders, Delhi.

7. Chemicals

Analytical grade potassium phosphate (monobasic), potassium phosphate (dibasic), sodium citrate, sodium carbonate, sodium chloride, copper sulphate, sodium potassium tartrate, sodium hydroxide, sodium hydrogen phosphate, phosphoric acid, lithium sulphate, sodium tungstate, sodium molybdate, glucose, magnesium chloride, hydrochloric acid, phenol, calcium chloride, sulphuric acid were purchased from B.D.H. (India). Tannic acid was purchased from W.J. Bush & Co., England. Methanol, Ethyl alcohol and solvent ether from Merck & Co. Ltd., (India) and bovine serum albumin was purchased from Sigma Chemical Co., U.S.A.

METHODS

METHODS

1. ISOLATION OF YEAST GLUCAN

Particulate glucan was prepared from compressed bakers yeast, S. cerevisae, by a modification of the method of Di Luzio et al. (1979). Since our modification of the method has not been published, the procedure is briefly described below:

About 540 grams of compressed bakers yeast was suspended in a 6 liter flask with 3 liter of 6% (w/v) aqueous sodium hydroxide solution. The suspension was placed in a boiling water bath for 4 hours, cooled overnight and then the dark brown supernatant was decanted. The insoluble material was then collected by centrifugation at 1200 g for 15 minutes. This procedure was repeated twice. The residue was then suspended in 3 liter of 3% (w/v) aqueous sodium hydroxide solution. The suspension was placed in a boiling water bath for 4 hours then allowed to cool. The suspension was stirred for 16 hours at room temperature. After dilution with 2 liter of distilled water, part of supernatant was decanted and the insoluble material was again collected by centrifugation at 1200 g for 15 min. This procedure was also repeated twice. The residue was then acidified with 300 ml of concentrated hydrochloric

acid plus 3 liter of 3% hydrochloric acid and placed in a boiling water bath for 6 hours. The suspension was allowed to stand overnight and the supernatant was decanted. The residue was further digested with 4 liter of 3% hydrochloric acid at 100°C for 4 hours, cooled overnight and decanted. The residue was washed six times with distilled water (20°C) and twice with distilled water at 100°C. Two liters of ethyl alcohol was added to the residue, mixed thoroughly and allowed to stand for a period of 48 hours for maximum extraction. This suspension was then filtered through a buchner funnel fitted with a Whatman filter paper of pore size 0.45 µm. The dark reddish brown alcohol filtrate was discarded. The residue was than suspended in ether and filtered through buchner funnel with 0.45 µm Whatman filter paper while the filtrate was discarded. The light brown residue was suspended in 2 liter ethyl alcohol and again filtered through buchner funnel with 0.45 µm Whatman filter paper by suction. The above process for obtaining alcohol supernatant was repeated until the alcohol supernatant was essentially colourless. The alcohol was removed by washing the residue four times with hot water. The light brown coloured particulate glucan preparation was then collected by centrifugation and suspended in 5% (w/v) dextrose. The particulate glucan was diluted to the desired concentration from stock solutions, placed in injection vials

and sterilized by autoclaving for 20 min. at 15 lbs. pressure and 120°C.

2. DETERMINATION OF MELTING POINT

Determination of melting point of solids plays an important role in the identification of organic compounds. A pure organic substance has a sharp melting point and the change from the solid to liquid state takes place within a range of 1°C. Presence of even a trace of impurity materially influences and lowers its melting point.

Thiele's method is generally used for determining the melting point of organic compounds. A small quantity of the organic substance is dried and finely powdered on a porous plate and introduced into a thin walled capillary tube about two inches long and sealed at one end. The solid is introduced into the capillary tube by first introducing the open end of capillary tube in the powdered compound, when some of it enters the capillary tube then the sample is carefully tapped to the end of the porous plate. The process is repeated two or three times so that the height of compound in the capillary is about half a centimeter.

In this experiment, the apparatus consisted of a 50 c.c. wide mouthed test tube containing conc. sulphuric acid and

fitted with a thermometer through the length of the cork. Then the bulb of the thermometer was moistened with conc. sulphuric acid and the capillary was placed close to the thermometer in such a way that the solid portion in the capillary was sticking to the bulb of the thermometer. The thermometer along with the capillary tube was introduced in the test tube in such a way that the closed end of capillary remained below the surface of conc. sulphuric acid and the open end of the tube remained above it. The test tube containing sulphuric acid was carefully heated uniformly through its surface by rotating the spirit lamp. The rise of mercury in the thermometer was noted from time to time. The temperature at which the Glucan sample melted to a clear liquid, was taken as its melting point. After noting the melting point the apparatus was allowed to cool at room temperature.

3. CARBOHYDRATE ESTIMATION

Carbohydrate content of our glucan sample was estimated by using the method of Dubois et al. (1956). Glucose solution was used as the standard.

(A) Preparation of standard solution:

10 mg of glucose was dissolved in 100 ml of double distilled water.

(B) Preparation of Phenol Reagent:

Phenol reagent was prepared by dissolving 8 gm of phenol in 100 ml of double distilled water.

An 0.1 ml of immunomodulator sample, glucan or glucose solution, was brought upto 2 ml volume by the addition of distilled water. Then 1 ml of freshly prepared phenol reagent was added. Later, 5 ml of concentrated sulfuric acid (95.5%) was added to the above mixture. The tubes were shaken vigorously and cooled at room temperature. The colour intensity was read at a wave length of 490 nm by using a Bausch and Lomb Spectronic-21 spectrophotometer.

4. MEASUREMENT OF pH

An ELICO L_{1-120} pH meter was used for all pH measurements. Sodium tetraborate (0.1 M, pH 9.18) and potassium hydrogen phosphate (0.05 M, pH 4.0) were used as standard buffer solutions to calibrate the pH meter.

5. HYDROLYSIS

Hydrolysis was carried out to determine the configuration of glucose units in the polysaccharide glucan. During hydrolysis of the polysaccharide, 1 gm portions of the test material were placed in 3 flasks and dissolved with fuming hydrochloric

acid at $0^{o}C$ for 24 hours, diluted with 100 c.c of distilled water and heated on a steam bath for one hour. The solution was then cooled and diluted to 200 c.c with distilled water and filtered before taking the polarimetric readings. The specific rotations of the solution were taken at one, two and five hour intervals using a polarimeter (ASCO Scientific Instruments, India).

6. TOXICITY TEST

It is essential to evaluate the potential toxicity of an adjuvant or immunomodulator before being brought into any therapeutic use. The toxic responses differ from species to species. It is not advisable to use a homogeneous strain (inbred strain) in toxicity tests, as the aim is to study new and unexpected effects of a substance in groups of animals of wider variability like random bred animals. A toxic effect that is seen in an animal model probably involves a common physiologic mechanism that is likely to be present in the humans, whereas such observations in one single species only indicates a particular effect in that species and is less likely to be present in another species.

Generally, a fifty per cent end point experiment is done to determine the minimal lethal dose of the substance.

In toxicity determination experiments, the lethal dose is that amount of a substance which kills on an average about 50 per cent of the test animals. The determination of LD 50 doses of a substance involves administering different dose concentrations to several groups of animals and observing the number of animals in each group which are killed within 48 hours of such administration. It is customary to administer doses in an increasing logarithmic ratio (successively doubled doses). Such doubling doses are given to each of the several animals which make up the experimental group. These experiments are so planned as to cover a complete range from a death to 100 per cent kill. But this is not the most economical use of the animals and if approximate toxicity of the preparation is known then the animals are divided into several groups, each receiving a dose supposed to be near or somewhat above/below the 50 per cent end point. Such a protocol fairly certainly ensures that in some groups the mortality will be higher, while in other groups it would be less than 50 per cent. In any screening program, toxicity tests on mice are usually performed first.

In the present experiment 10 to 12 weeks old mice, each weighing between 12 to 15 gm were used. Mice were housed in metal cages and fed on pellet diet (Hindustan Lever Ltd.,India).

All mice were quarantined and acclimatised to laboratory conditions for at least seven days before experimentation. During this observation peroid, the mice which were less active or showed any other sign of abnormality were excluded from the study. The mice were divided into groups of five each and were placed in separate cages, mainly to select the dose ranges for the subsequent study. The glucan was administered through a single intraperitonial inoculation. The dosages selected were 0.25 mg, 0.5 mg, 1.0 mg, 2.0 mg, 4.0 mg, 8.0 mg, 16.0 mg, 32.0 mg, 64.0 mg and 128.0 mg respectively. The injected mice were observed continuously for two hours and then occasionally for further four hours, and were finally left overnight following which the mortality was recorded. One group of mice received only one single dose. One group containing 5 mice were kept as a control. Any test dose killing 50 per cent of mice in a group, was taken as an LD 50 dose.

7. ADJUVANT DOSE RESPONSE STUDY

To carry out the study of adjuvant dose response, rabbits were immunized with glucan in different concentrations. Sixteen rabbits were used in the experiment. All rabbits were quarantined and acclaimatised to laboratory conditions for at least one week before immunization. Rabbits were fed a pellet diet (Hindustan Lever Ltd., India) ad libitum. They had free

access to water. During this observation period, the animals which were less active or showed any other abnormality, were excluded from the study. The rabbits were then divided into four groups of four each and were placed in separate cages for subsequent immunization experiments. The rabbits in the first group were inoculated with 5% (w/v) dextrose only and were used as controls. The rabbit in the second, third and fourth groups were immunized, intraperitoneally, with 20 mg, 40 mg and 60 mg concentrations of glucan respectively. Three doses were given in three weeks while fourth week was the rest period. The fourth and the last dose inoculated in the fifth week was the booster dose. The entire immunization schedule was completed over a five week period in a total of 4 doses. The antisera obtained from the immunized animals were used for checking the presence of specific antibodies against glucan samples. The immunization schedule is shown in Table I.

8. INDIRECT HAEMAGGLUTINATION TEST (IHA)

Indirect haemagglutination tests were carried out in the sera of immunized animals for checking the antigenicity of glucan. This test was done according to the method of Mathews et al. (1975) with few modifications. Microplates with U shaped bottom (Cook U-Plate, Cook Engineering Co.,

TABLE - I: IMMUNIZATION SCHEDULE.

Animal Groups	No. of Rabbits	Inoculations	Weekly Immunization dose (ml)			
			Ist	IInd	IIIrd	Vth
I (Control)	4	5% (w/v) Dextrose	1.0	1.0	1.0	1.0
II (Experimental)	4	20 mg/ml Glucan	1.0	1.0	1.0	1.0
III (Experimental)	4	40 mg/ml Glucan	1.0	1.0	1.0	1.0
IV (Experimental)	4	60 mg/ml Glucan	1.0	1.0	1.0	1.0

*Fourth week was treated as rest period.

All injections are given intraperitonially.

Total inoculum in each injection was 1 ml.

Alexandria, Virginia) were used for this test. Sheep red blood cells were washed four times in isotonic phosphate buffer saline, pH 7.2 by centrifugation for ten minutes at 1500 rpm. A 3% suspension of sheep RBC was prepared in isotonic phosphate buffer saline. The diluted RBC suspension was mixed with an equal volume of 1/20,000 tannic acid and incubated at 4°C for 30 minutes with intermittent shaking. After incubation the suspension was centrifuged at 1200 rpm for 10 minutes and tanned sheep RBC's were washed three times with phosphate buffer saline containing 0.5% bovine serum albumin (BSA). The above was resuspended in phosphate buffer saline containing bovine serum albumin to obtain a final concentration of 3% (v/v). The tanned sheep RBCs were sensitized with an equal volume of diluted glucan and incubated at 37°C for 30 minutes with intermittent shaking for obtaining a uniform antigen coating of RBCs. After incubation they were centrifuged to remove the uncoated antigen supernatant. The pellet was washed 3-4 times with phosphate buffer saline containing bovine serum albumin. An 1.5% (v/v) antigen coated RBC suspension was finally prepared for using it in the tests.

To each well of the 'U' bottomed microtiter plate, 0.025 ml of a serially diluted glucan antiserum was added along with an equal volume of antigen coated sheep RBC suspension. The plates were sealed with transparent gummed tape and gently

shaken for 5 minutes at room temperature. The plates were incubated at room temperature in humid chambers for 1 hour and then kept overnight at $4^{o}C$. The highest dilution of the test serum giving a positive carpet like pattern was recorded as the titration end point. Negative reactions were detected by the formation of a compact button with entire margin.

RESULTS

RESULTS

ISOLATION OF β-1,3 GLUCAN

β-1,3 Glucan, a cell wall component of S. cerevisae, was extracted to obtain a better yield and purified. The procedure devised for isolation of β-1,3 Glucan resulted in an overall yield of 7.4 per cent of the weight of compressed yeast. The yield was somewhat on the lower side owing to inadvertent experimental losses during fractionation. During fractionation with ethyl alcohol, some oily droplets were observed in the filtrate. A plot depicting the comparative yield of glucan obtained by earlier workers is shown in Figure 1.

MELTING POINT

The temperature at which the isolated glucan began to melt to a clear liquid was read as 131°C. This was taken as the melting point of the glucan extracted in this study. The melting point of glucan as determined in this and other studies is shown in Figure 2.

CARBOHYDRATE CONCENTRATION

The carbohydrate concentration of the isolated glucan, estimated by the method of Dubois et al. (1956) was estimated as 240 mg/ml. Carbohydrate estimation graph is shown in Figure 3.

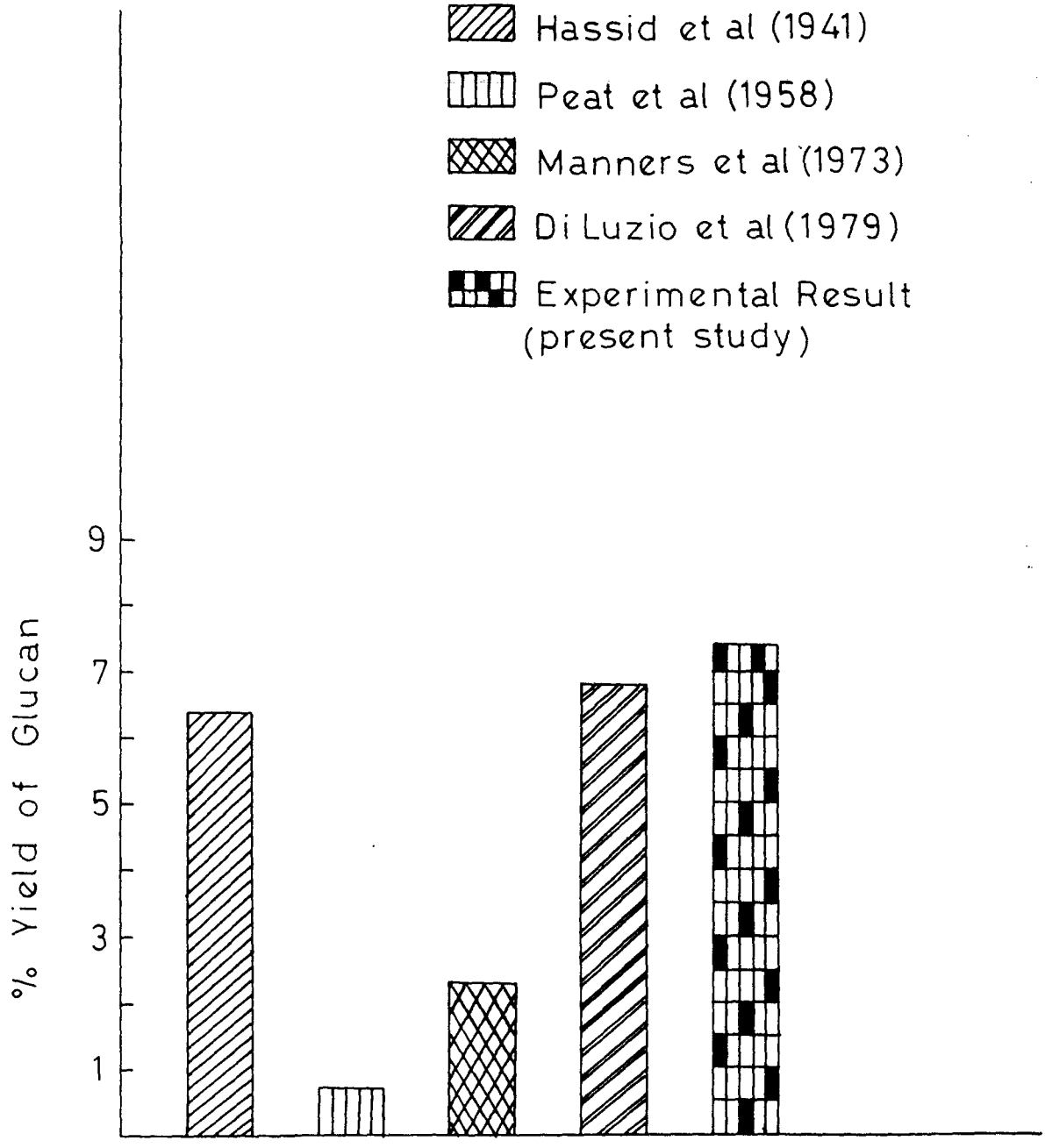


FIG. 1 – COMPARATIVE YIELD OF GLUCAN FROM S. CEREVISAE

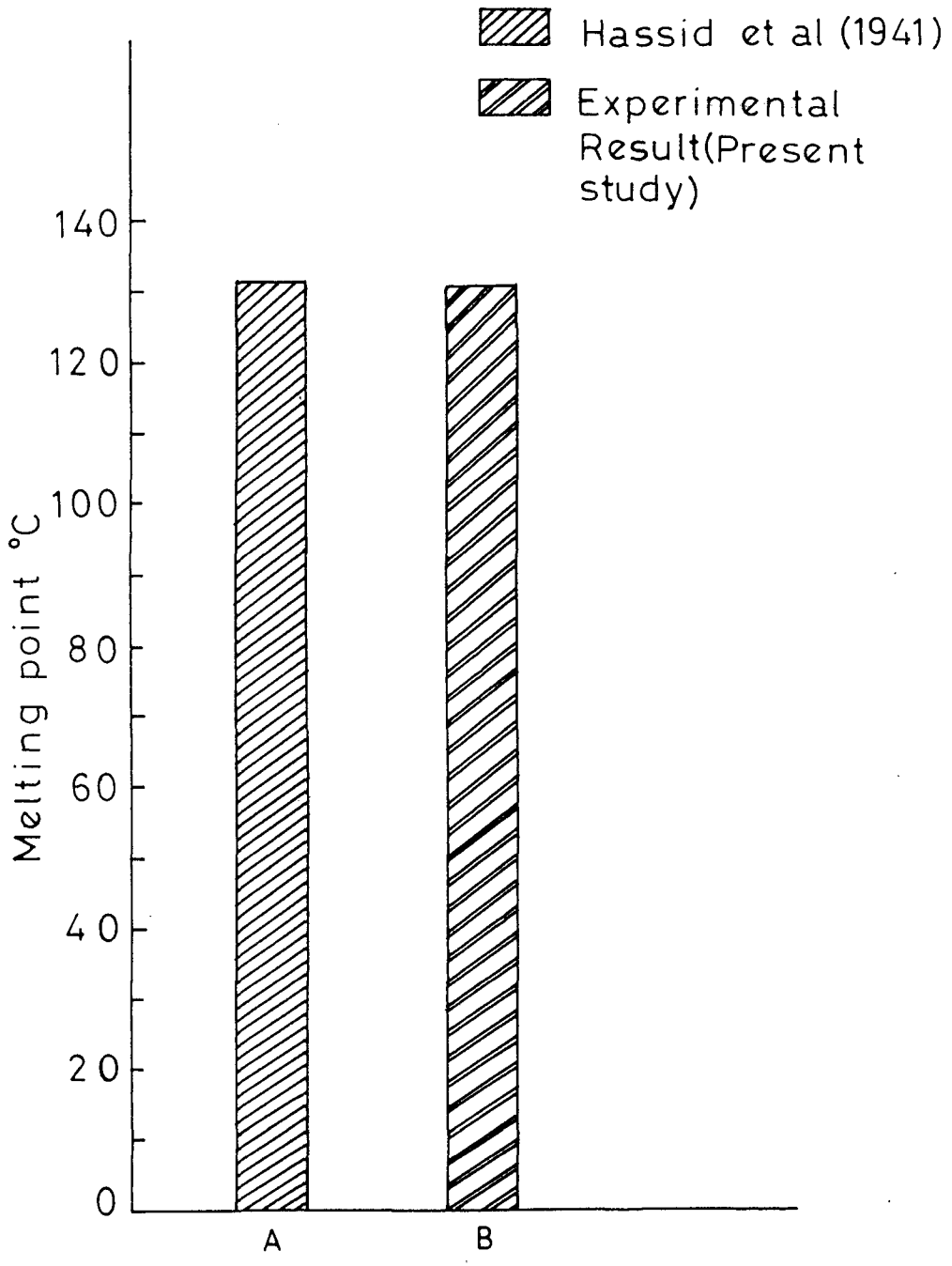
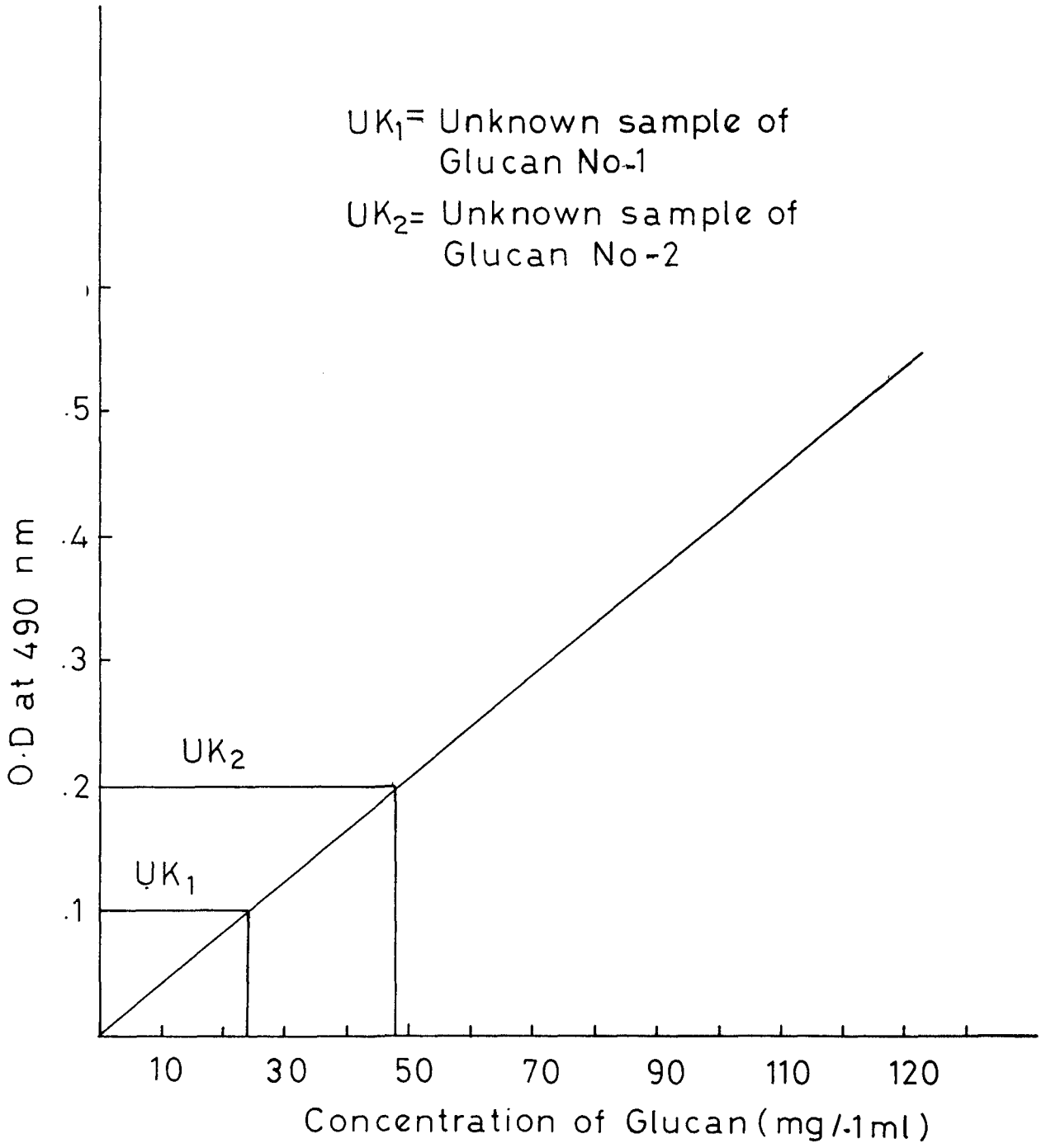


FIG.2 - COMPARATIVE MELTING POINT OF GLUCAN



FIG·3-CARBOHYDRATE CONCENTRATION CURVE

(DUBOIS et al, 1956)

HYDROLYSIS

The specific rotations of the diluted hydrolysed polysaccharide solutions were taken at one, two and five hour intervals. The specific rotations, $(\alpha)_D$ at different intervals were $+0.47^{\circ}$, $+1.02^{\circ}$ and $+1.50^{\circ}$ respectively. The upward mutorotation during hydrolysis indicated a β-configuration of the glucose units in the polysaccharide glucan.

TOXICITY TEST

The data, mortality graph and animal titration results, presented in this study was analyzed as follows. The dose given in milligrams to an experimental animal were converted into log dose and plotted along the X-axis on a graph paper. Per cent mortality in each group at various doses was converted into probits and plotted along the abscissa. The curve was extrapolated according to the method of least squares. The LD 50 was obtained from the graph by finding out the log dose at probit 5. The experimental curve gave an LD 50 dose at 31.6 mg. Thus for a mice the LD 50 dose was greater than 2 g/kg upon intraperitonial inoculation. The log-dose probit response for mortality per cent lethal response to glucan is shown in Figure 4.

TABLE II

Animal Titration of a Toxin/ Immunomodulator Glucan.

Dose (mg)	Total No. of mice	Survivor (Number)	Dead (Number)	Percent mortality
0.25	5	5	0	0
0.5	5	5	0	0
1.0	5	4	1	20
2.0	5	5	0	0
4.0	5	5	0	0
8.0	5	4	1	20
16.0	5	4	1	20
32.0	5	3	2	40
64.0	5	1	4	80
128.0	5	0	5	100

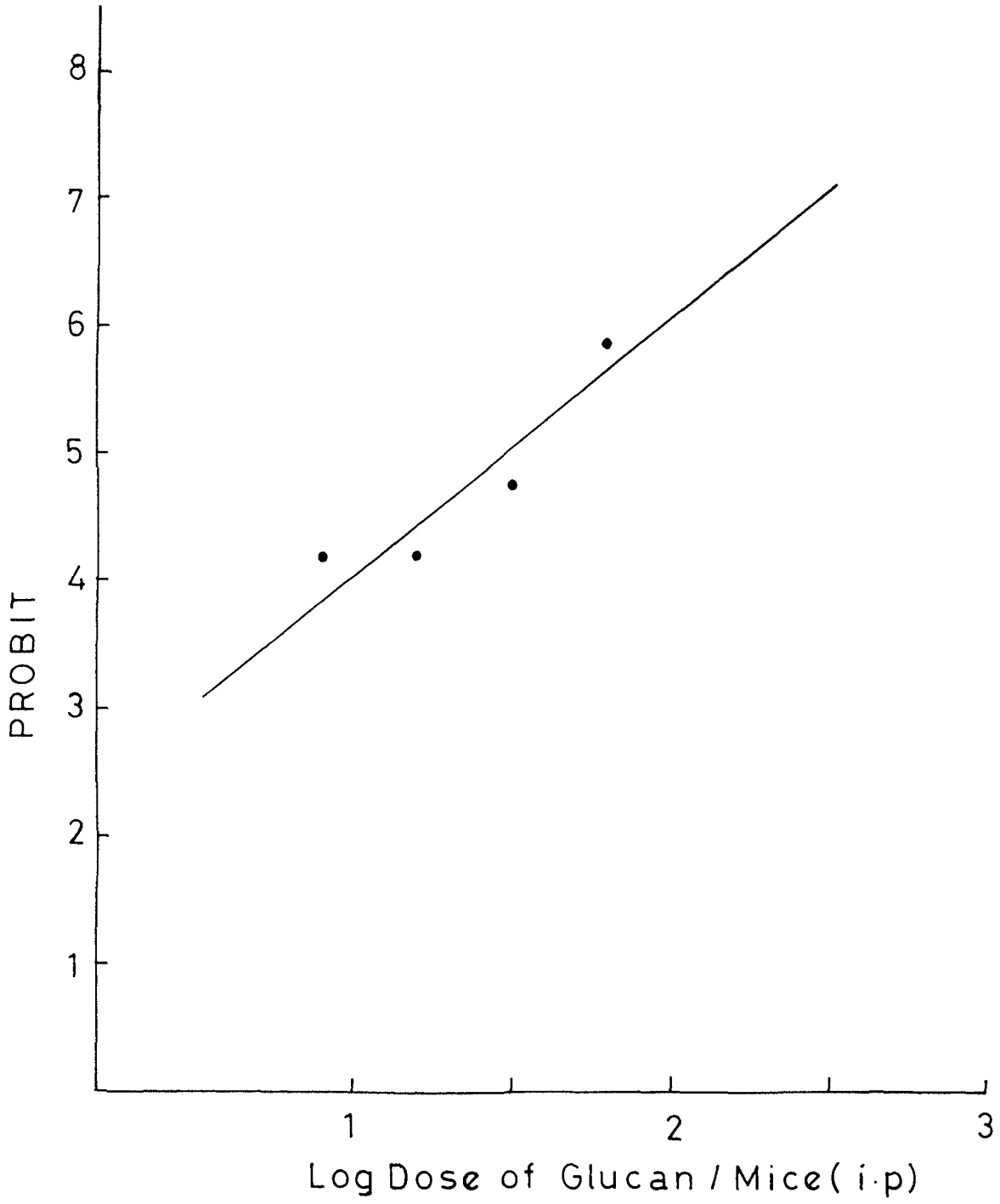


FIG. 4 - LOG DOSE PROBIT RESPONSE FOR MORTALITY PERCENT LETHAL RESPONSE TO GLUCAN

INDIRECT HAEMAGGLUTINATION(IHA) TEST

Rabbits immunized with glucan showed markedly increased antibody levels compared to control rabbits given only 5% (w/v) dextrose. The highest IHA titers in experimental group III inoculated with glucan at 40 mg/kg body weight was recorded in the fourth and the sixth week. Group III animals showed approximately a 3 fold increase in antibody level compared to group IV. Serum samples of control rabbits given only 5% (w/v) dextrose were negative. A titer value of less than 64 was considered negative. The detailed IHA results are shown in Table III.

TABLE III

IHA Titers in Rabbit Sera

Animal Groups	IHA Titers	
	4th week	6th week
II (20 mg/kg Glucan)	1 : 512	1 : 1024
III (40 mg/kg Glucan)	1 : 4096	1 :16384
IV (60 mg/kg Glucan)	1 : 2048	1 : 4096

An IHA Titer less than 64 was taken as negative.

DISCUSSION
&
CONCLUSION

DISCUSSION

Glucan, a β-1,3 polyglucose isolated from the inner cell wall of S. cerevisae, is a well recognized immunomodulating agent (Di Luzio et al. 1963, 1976). Glucan for its immunomodulating role has been a subject of continued interest and study for the past several years. The investigations and the findings presented in this thesis are chiefly based on studies pertaining to the isolation, toxicity and adjuvanticity of β-1,3 Glucan. Such studies relating to the toxicity of glucan are not too many as to allow a wide comparison of the numerous reports claiming its efficacy in the stimulation of reticuloendothelial system, immunologic and hemopoietic functions. All previous reports show disparities in the biological activity of various glucan preparations. In particular, the effect of glucan has been reported to depend more on the batch (lot) and/or source used (Suit et al. 1978). In this study glucan was prepared as per the modified method of Di Luzio et al. (1979) and Hassid et al. (1941). An attempt was made to standardize the preparation for obtaining a sample with uniform immunobiological activity within the various lots. It was considered necessary to eliminate the problem of glucan-associated biological variance. Such standardization is essential in view of its

possible use in future vaccination studies, as also for obtaining consistently uniform results in any future attempts towards development of an effective vaccine against parasitic infections. The usual practice in the past was that most of the investigators were able to obtain glucan from one or two laboratories in Europe and United States for carrying out the immune studies against parasitic infections such as Amoebiasis, Leishmaniasis, etc.

But more recently numerous attempts have been made to isolate glucan from S. cerevisae for use in various laboratories. Hassid et al. in 1941 were the first to isolate glucan with a total yield of 6.4% of the weight of the compressed yeast. Peat et al. in 1958 made an attempt to isolate glucan from S. cerevisae. The yield obtained was very low of about 0.65% of the weight of compressed yeast. Then Manners et al. (1973) isolated glucan from S. cerevisae and the yield obtained was 2.4% of the weight of compressed yeast. Finally, in 1979 Di Luzio et al. with the modification of the procedure of Hassid et al. were able to isolate glucan with a better yield 6.8% of the weight of compressed yeast. Our results clearly indicate that glucan can be successfully isolated for individual use in laboratories by adopting certain modifications in the procedure employed by previous

workers. We were also able to increase the yield to a certain extent. The yield of glucan in this study was 7.4%. This yield was approximately 0.6% higher than what Di Luzio et al. (1979) obtained using their procedure. The exact reason for this difference in the yield may be due to the repetition of a few steps and certain modifications such as employing buchner funnel fitted with 0.45 µm Whatman filter paper during filtration by suction. In addition to this, we employed a procedure for alcohol extraction of glucan with ethyl alcohol and this was repeated until the alcohol filtrate was essentially colourless and no oily droplets were seen. By repeating this step, there was a complete elimination of organic solvent soluble substances. The use of 0.45 µm Whatman filter paper has saved inadvertent experimental losses of glucan particulate during fractionation.

After estimating the melting point of the isolated glucan as 131°C , chemical analysis was carried out for the total carbohydrate content according to the procedure of Dubois et al. (1956). The confirmation of β-configuration of glucose units in the isolated polysaccharide glucan was assayed by reading specific rotations (α) D during hydrolysis at different intervals. The upward mutorotation indicated a β-configuration. The results thus reported were found to be in complete agreement with the results obtained by earlier

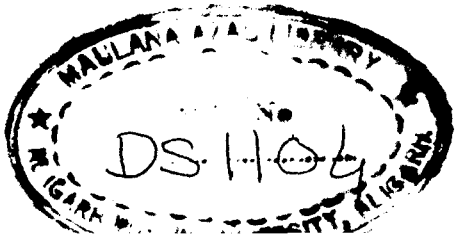
workers such as Hassid et al. (1941), Peat et al. (1958) and Manners et al. (1973).

These studies were also focused on the evaluation of any available toxicity of the isolated glucan. Such a study was undertaken because of the reported differences in the biological activity of various glucan preparations depending on the batch/lot and/or the source material used. Surprisingly, few therapeutic indices have been recorded to date, and it is almost impossible to establish the toxicity of some of these compounds. The simplest estimation of toxicity, which involves recording of change in the body weight over a short period following adjuvant administration are rarely if ever reported. Unfortunately, this information cannot be established readily from published LD 50 data, as almost invariably several routes of administration or varying conditions of stress have been employed to determine the lethal dose. The nature of the animal species studied is very important in this context. FCA has also been a widely used immunoadjuvant. The FCA treated animals were found more predisposed to skin lesions. Histologically, perivascular cuffing with mononuclear cells were found in the initial phase (Freund, 1953). More recently, the toxic properties of cord factor has been studied to a certain extent. Cord factor is toxic for mice

when administered in 10% emulsions of paraffin oil in repeated injections. Toxicity of cord factor depended on the size distribution of oil droplets (Bekierkunst, 1977). In vivo and in vitro studies indicate that cord factor causes the structural disintegration of mitochondrial membrane, leading to specific inhibition of oxidative phosphorylation (Kato et al. 1978). This study thus eliminated the doubts regarding glucan-associated immunobiological variance, if any, for its future use in vaccination studies which must be carried out in order to evaluate its potential effectiveness. One benefit of such a study was to reduce the risk of lethality generally believed to be exerted by glucan to the immune system following immunization. This study also helped in establishing a correct dose pattern to evaluate the adjuvanticity. However, such observations in one species only indicate a particular effect in that species and less likely to be present in another species. Our results clearly indicate that the LD 50 dose of glucan, i.e. the dose of glucan which would kill on an average 50 per cent of the test animals, was found to be greater than 2 gm/kg for mice when given intraperitoneally. This LD 50 value reflects the limit range for the safe use of glucan. The LD 50 values obtained here are in agreement with the results obtained by Patchen et al. (1980, 1983) and Cook et al. (1980).

The extensive and varied review regarding the broad spectrum activity of immunopharmacologic activities of adjuvant glucans are well documented. These and other similar studies only demonstrate the growing interest in these unique molecules. Since the end metabolite is glucose, a new dimension in pharmacology and therapeutic uses employing this newer molecule is clearly indicated. Glucan has been shown to have significant effects upon humoral immunity (Wooles and Di Luzio, 1962) and cell mediated immunity (Wooles and Di Luzio, 1962). Administration of glucan is usually associated with enhancement of macrophage/monocyte cell series, with a corresponding increase in the activity of reticuloendothelial system (Wooles and Di Luzio, 1963). Glucan has been shown to enhance host resistance to a variety of experimentally induced bacterial (Reynolds et al., 1980), fungal (Williams et al., 1978), viral (Reynolds et al., 1980; Williams and Di Luzio, 1980) and parasitic (Cook et al., 1980) diseases. It was also shown that intraperitoneal administration of glucan significantly modified the course of *E. coli* sepsis in mice (Williams et al., 1983). Glucan was also found to exhibit a strong adjuvant effect when inoculated intravenously with killed erythrocytic stages of *P. berghei*. Mice pretreated with dead parasite and glucan showed 100 per cent survival after challenge, as opposed to 29 per cent survival of mice pretreated with dead parasites. Holbrook et al. (1981)

through their studies demonstrated that the mice which received glucan only were all dead following challenge. Patchen and her group have extensively studied the influence of glucan on murine pluripotent stem cells, and on myeloid and erythroid progenitor cells. In a series of extensive studies, Patchen demonstrated that following i.v. administration of glucan in the dose range of 0.1-2.0 mg per mouse, the proliferation of bone marrow and splenic pluripotent stem cells, splenic macrophage and granulocyte-macrophage colony forming cells, and splenic erythroid colony and burst-forming cells were stimulated in a direct, dose dependent manner. The molecular mechanisms through which glucan mediates its stimulatory effects on hemopoiesis are still largely unknown (Patchen et al., 1983). But now there are increasing evidences to suggest that immune enhancement and protection by glucan is macrophage mediated. Immuno-amplification by glucan is further supported by the data obtained from experiments showing complete loss of protection to E. coli infection in glucan treated mice which were subsequently given a macrophage suppressant (William et al., 1983). Deimann and Fahimi (1979) observed that glucan induced a dose dependent accumulation of macrophages in the liver. By scanning electron microscopy, as well as histochemistry, the derivation of kupffer cells, which are present in massive



numbers in the liver of glucan - treated animals was thought to arise mainly from bone marrow and to a minor extent through self replication *in situ*. In our studies for assessing the adjuvanticity of isolated glucan, the rabbits were inoculated with 20 mg/kg, 40 mg/kg and 60 mg/kg doses respectively. A total of four doses were given in five weeks time. Our results clearly indicate that the antibody titers as evaluated by IHA were found to be highest with 40 mg/kg dose. The results thus obtained correspond with the work of Patchen et al. (1983) on dose dependent responses of immunomodulating agent , Glucan.

While concluding the results based on this study, we can say that the isolated material from bakers yeast, *S. cerevisae*, is a polyglucose β-1,3 glucan, capable of enhancing the immune responses in a variety of host animals. The results of this study are comparable with the results of numerous other workers who used glucan in their researches. Ultimately, such studies will be helpful in eliminating the doubts regarding glucan associated biological variance as glucan is going to be increasingly used in the coming years for enhancing the immune responsiveness against a large number of parasitic antigens in susceptible hosts.

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